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FOREWORD

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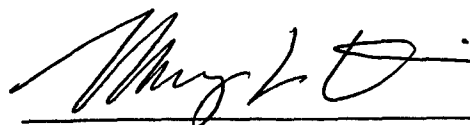
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INTRODUCTION:

The HER-2/neu (HER2) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. HER2 over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. HER2 may also be related to cancer formation, with overexpression being detectable in 50-60% of ductal carcinomas in situ (DCIS).

The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. Preliminary studies prior to the grant discovered that some patients with breast cancer have existent CD4+ helper T cell immunity and antibody-mediated immunity to HER2. HER2 is a self protein. Therefore, before our studies it had been assumed that patients would be immunologically tolerant to HER2 and that immunity could not be generated. Our prior studies demonstrating that immunity is already present in some patients with breast cancer implied that immunity to HER2 is induced in some individuals by virtue of the presence of growing cancer expressing the antigen and gives credence to the concept that HER2-specific immunity can potentially be used in therapy without destroying normal tissue. The current grant is exploring issues important for developing HER2 specific vaccines and T cell therapy. In addition, the demonstration of immunity to HER2 offers the opportunity to explore host-tumor interactions in a well-defined antigen system.

Specific Aim #1 is examining Ab immunity to HER2. Preliminary data showed that Ab immunity to HER2 can be detected in the sera of some patients with breast cancer. Studies were proposed to determine the frequency of Ab immunity, the relative frequency of functional Ab and to determine whether responses to HER2 are beneficial or detrimental. Additional studies were proposed to determine whether immunity to HER2 can serve as a marker for early cancer and/or whether changes in level represent a marker for relapse.

Specific Aim # 2 is examining CD4+ T cell immunity to HER2. Preliminary data showed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy. In animal models CD4+ T cells can be effective against abundant soluble proteins. The extracellular domain (ECD) of HER2 is shed abundantly in some patients. Studies were proposed to determine the prevalence of CD4+ responses in patients with HER2+ tumors and to determine whether changes in immunity occur with therapy and relapse.

One limitation to the development of human anti-cancer vaccines and T cell therapy is that determination of immunogenicity conventionally requires immunization in vivo. Therefore, much effort was proposed to develop methods of priming in vitro. Preliminary data in the grant proposal described prior studies developing a culture system using dendritic antigen presenting cells that allows exceedingly rapid priming in vitro. Studies were proposed to determine whether the priming system is reproducible enough and powerful enough to allow determination of which peptides are immunogenic and which peptide specific T cells are capable of responding to whole HER2 protein. Identification of epitopes is important for eventual incorporation into peptide based vaccines or for use to stimulate T cells in vitro for T cell therapy regimens.

Specific Aim # 3 is examining CD8+ CTL immunity to HER2. Preliminary data for the grant showed that CD8+ CTL can be primed to HER2 peptides in vitro and that primed peptide specific CTL can lyse HER2 positive cancer cells. However, the systems employed are extremely fastidious. In vitro priming was to be developed and used to identify the immunogenic epitopes of HER2. Additional studies were proposed to determine whether patients with breast cancer have existent CTL immunity to HER2, as had previously been described for patients with ovarian cancer. For patients with CTL immunity and HER2 positive cancers, studies were proposed to determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of

immunity with therapy and relapse. Finally, studies were proposed to determine whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor and can be expanded in vitro to the extent needed for adoptive therapy.

BODY:

The BODY of the Progress Report will be organized according to the STATEMENT OF WORK in the original grant proposal.

Specific Aim #1: To examine Ab immunity to HER-2/neu.

Task 1: Months 1-48 To determine the frequency of Ab, the biologic function of HER2 specific Ab, Ab correlation with HER2 overexpression and Ab correlation with circulating ECD.

Determining the frequency of Ab to HER2 required first obtaining adequate sera from well-defined patient populations. Prior studies had been performed with sera drawn at random times in the breast cancer course. Several studies are currently underway analyzing sera drawn at the time of diagnosis in well-characterized patients. One study has been published to date and described in the last progress report. This study evaluated HER2 antibodies in breast cancer patients [Disis et al JCO 1997]. Evaluations were performed on sera drawn at the time of diagnosis from patients with early and late stage breast cancer and revealed a strong correlation between HER2 specific immunity and HER2 overexpression in the primary tumor. Two further studies have been completed, one in HER2 positive colon cancer and one in lung cancer. Sera, drawn at the time of diagnosis were available from both patient populations and allowed us to address the issue of HER2 as a shared tumor antigen among many solid tumor types. Data derived from these studies lend credence to the hypothesis that HER2 specific immunotherapy would be effective in the eradication of HER2 overexpressing disease from any tissue specific site, not just breast cancer. Recently, a more quantitative assay for measuring antibodies to HER2 has been developed. This ELISA based evaluation also allows the determination of the Ig class involved in the HER2 antibody response. In collaboration with Dr. Robyn Ward [Ward et al submitted], sera from patients with HER2 positive and negative colon cancer were analyzed. Both IgG and IgA HER2 specific antibodies were detected. In addition, the presence of HER2 antibodies correlated strongly ($p < 0.01$) with the presence of HER2 protein overexpression in the primary tumor. Finally, quantitation of the HER2 antibody response allowed some comparison with antibodies derived

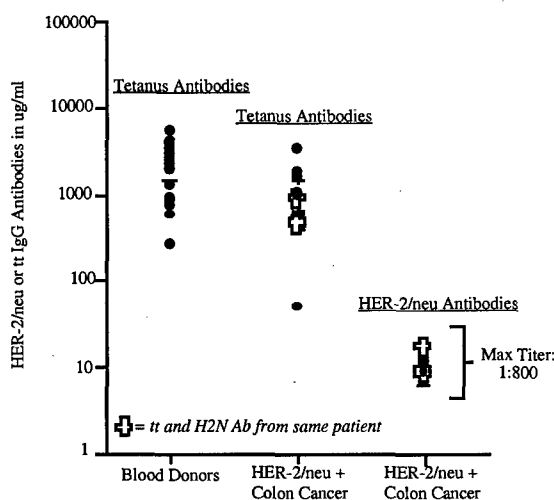


Figure 1. HER2 antibodies are detected at a much lower level than tetanus antibodies. IgG antibodies to tt or HER2 were measured by ELISA. Volunteer blood donors were used as the control population. The open crosses represent tt and HER2 antibodies detected in the same patient.

from a classic immunization, such as tetanus toxoid (tt). Fig. 1 shows that HER2 antibodies are present at much lower levels than tt specific antibodies. This lack of magnitude of the tumor specific antibody response is not due solely to potential immunosuppression by the colon cancer. Three patients had been recently immunized to tt while they had cancer and their tt specific antibody levels were at the same level as those found in volunteer blood donors. Antibodies induced by "immunization" with their HER2 positive tumor, however, were found at much lower levels. Thus, one of the potential problems with the HER2 specific antibody response may lack of magnitude of that response.

A second study performed in 29 patients with non-small cell lung cancer evaluated both HER2 specific antibodies and HER2 ECD circulating in the sera as a potential tumor marker. 200 normal volunteer blood donors were used as the control population. A positive HER2 antibody response was defined as a titer greater than or equal to 1:100. 8/29 lung cancer patients had HER2 titer greater than or equal

to 1:100. None of the volunteer blood donors did. Thus, the specificity of this assay was 100%, although the sensitivity of the assay in detecting lung cancer was only 28%. All 8 of the patients who had antibodies had HER2 overexpressing primary lung cancers as measured by IHC. Serum circulating ECD was also measured. There was no correlation between presence or lack of antibodies and the presence of circulating ECD. However, HER2 antibodies were more likely to be found in early stage patients (I/II) while circulating ECD was more likely to be elevated in Stage III/IV patients.

Preliminary data from the last progress report revealed that HER2 immune sera could also be used to define novel tumor antigens. Two general approaches have been described to identify novel tumor proteins recognized by serum antibodies. The first, described in the previous report, involves a protein-based approach in which tumor cell proteins are run on one or two-dimensional polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with patient serum samples. Tumor proteins that are recognized by serum antibodies can then be excised from the gel and identified through peptide microsequencing. The second, a cDNA-based approach known as SEREX (serological identification of antigens by recombinant expression cloning), has recently been described by Sahin et al. In this strategy, RNA isolated from tumor cells is used to construct a lambda phage cDNA library for expression in bacterial cells. The library is plated on agar, transferred to nitrocellulose and immunoblotted with patient or normal serum (serum samples are first pre-cleared of antibodies to E.coli and phage proteins). Phage plaques that are recognized by patient serum antibodies, but not by serum antibodies from normal controls, are likely to contain cDNAs encoding immunogenic tumor proteins. These cDNAs can then be directly isolated from phage, sequenced by standard methods, and further evaluated in screening assays. SEREX is an established immunological technique used to identify novel immunogenic proteins. We are developing that technique to define novel antigens in early stage breast cancers for potential use as therapeutic immunologic targets. Using sera pre-immune for HER2 allows some internal positive controls to be built into the developmental system.

A critical requirement for successful SEREX screening is the depletion from serum samples of antibodies to E.coli, which are found at varying titers in the serum of all individuals. Indeed, the titer of antibodies to E.coli proteins is often greater than the titer of autoantibodies to the tumor antigens that are the objective of the SEREX screen. We and others have solved this problem by pre-incubating serum samples with protein lysates made from the same strain of E. coli used to plate the cDNA expression library. To do this, protein lysates from the E. coli strain Y1090⁻ are prepared by sonication and crosslinked to a cyanogen bromide activated CL-4B matrix (Pharmacia) according to the manufacturers protocol. The protein content in the supernatant is determined using a BCA protein assay kit (Pierce). Ten mg of protein is then added to 1 g of matrix (which had been pre-washed with 1mM HCl) and rocked overnight at 4°C.

To deplete serum of E.coli-specific antibodies, a 1 ml aliquot of frozen serum is thawed and centrifuged at maximum speed in a refrigerated microfuge for 20 minutes. Cleared serum is then mixed with 1 ml bed volume of cross-linked matrix, which had been washed with 5 ml of TBS (20 mM Tris, 0.5 M NaCl, 0.05% azide, pH 7.5), and rocked overnight. Serum is separated from the matrix by passage over a plastic column, and residual serum is recovered by washing the column with 5 ml TBS. The entire eluate is pooled for use in SEREX. The serum used to screen for novel antigens is that derived from breast cancer patients who already have a pre-existing immune response to HER2. The serum is depleted of E.coli-specific antibodies and used to screen 1×10^5 clones of a commercial lambda phage gt-11 cDNA expression library derived from the human breast cancer cell line ZR-75.1 (Clontech). To accomplish this, mid-log phase cultures of Y1090⁻ grown in 1 mM maltose are pelleted and resuspended in 10 mM MgSO₄. Cells are mixed with a volume of diluted library that is calculated to yield 5×10^3 pfu per 100 mm plate, and incubated at 37°C for 15 minutes. The infected bacteria are then plated onto 100 mm plates using NZY top agar and incubated for three hours at 37°C. Circular supported nitrocellulose membranes (Schleicher and Schuell) are soaked in 1 mM IPTG in sterile H₂O for 10 minutes and then air

dried. The membranes are placed on the surface of the bacterial plates when phage plaques first became visible. The orientation of each membrane is marked by stabbing an 18 g needle through the membrane and the underlying agar. After exposure to the plate for 1 hour, the membranes are carefully removed and placed with the bacterial side up on the surface of a second NZY plate (a feeder plate), and incubated overnight at 37°C. The use of a feeder plate is intended to allow multiple membrane lifts from the same master plate in future experiments.

After overnight growth, the membranes are removed from the feeder plates and washed once in TBST (Tris buffered saline containing 0.05% Tween-20) followed by two ten-minute washes in TBS. Membranes are blocked in TBS + 1% BSA (bovine serum albumin) for 2 hours at room temperature with continuous rocking. Membranes are then incubated overnight at room temperature with patient serum that had been pre-cleared of *E. coli*-specific antibodies and diluted 1:100 in TBS + 1% BSA. The following day, the membranes are washed three times with TBS, and then incubated for 45 minutes with alkaline phosphatase-conjugated goat anti-human antibody (Pierce) diluted 1:7500 in TBS + 1% BSA. The membranes are then washed three times in TBS and developed with NBT/BCIP (Nitro blue tetrazolium chloride at a final concentration of 330 ug/ml, and 5-bromo-4-chloro-3-indoyl phosphate at a final concentration of 165 ug/ml in a buffer composed of 100 mM NaCl, 5 mM MgCl₂ and 100 mM Tris, pH 9.5) for approximately 5 minutes. Immersing the lifts in distilled H₂O stops the color development. In initial experiments, using sera from a patient with high titer HER2 antibody (>1:800), a screen of 1 X 10⁵ pfu yielded thirteen plaques which demonstrated immunoreactivity with the patient serum sample. Of these 13, five have so far been subcloned and sequenced. Current studies are evaluating the immunogenicity of these novel proteins in larger populations with breast cancer and studies are continuing to identify novel antigens that elicit an immune response in the majority of breast cancer patients tested.

The presence of HER2 specific antibody responses in breast cancer patients and the correlation with HER2 positive cancer in early stage patients strongly implies that immunity to HER2 develops as a result of exposure of patients to HER2 protein expressed by autologous cancer. The correlation between antibody and breast cancer shows that antibody responses have the potential to serve as a tumor marker for detecting the breast cancer. More accurate means of measuring antibody, such as direct quantitation, may allow improved sensitivity and specificity of serum HER2 antibodies as a potential diagnostic or prognostic tool. Finally, sera from breast cancer patients can be used to define potentially novel breast cancer antigens. These findings should stimulate further studies to develop the use of immunity to oncogenic proteins as tumor markers and should stimulate the development and testing of vaccine strategies to induce and augment immunity to HER2 and other breast cancer related antigens for the treatment of breast cancer.

Task 2: Months 1-24 To examine biologic function of Ab binding to the ECD.

HER2 is a growth factor receptor. Molecules binding to the HER2 receptor, including the putative natural ligand(s), can have variable effects on stimulating malignant cells. Heterologous antibodies can have an array of effects upon binding to the HER2 extracellular region, with some inducing agonistic effects and some antagonistic effects. In order to evaluate and subsequently predict whether patient HER2 Ab correlates with clinical outcome, it will be necessary to determine whether some Ab function. If some Ab is functional, in order to study the correlation between Ab and outcome, it will be necessary to stratify patients as to whether their Ab is functional and the nature of that function. Preliminary data showed that several patients had Ab to the cysteine rich area of the ECD and, in one of the patients, that the Ab induced decreased protein phosphorylation of HER2+ breast cancer cells. Recombinant HER2 ECD and ICD have been constructed as described in the previous progress report and studies are currently underway screening patients for ECD specific HER antibody response to evaluate HER2 antibodies for their functionality.

The initial phase of these studies is to develop the assays to evaluate functionality of endogenous HER2 antibodies. We have obtained the alpha and beta heregulin egf domains from purified Genome Systems clones 49439 (beta) and 155975 (alpha) and have amplified the egf domains of human heregulin for insertion into pMal-c vector. These constructs will produce HER2 ligand for use in phosphorylation, binding and proliferation assays as a native control. In addition, we have developed a cell inhibition assay using NIH 3T3 cells, which have been transfected with the human HER2 cDNA. Parental NIH 3T3 cells can be used as a control cell in these assays. Briefly, control or experimental cells are plated at 1×10^4 cells/well in RPMI and 10% FCS. Varying concentrations of human purified Ig which have been shown to contain HER2 ECD specific antibodies are added in varying concentrations. A control well incubated with AG879, a kinase inhibitor, is used to determine maximal inhibition. To date 7 patients have been evaluated for the ability to selectively inhibit the proliferation of HER2 transfected, but not parental cells (HER2-) in culture using tritiated thymidine incorporation assays. 3/7 patient sera demonstrate specific cellular growth inhibition of more than 2 standard deviation below untreated cells. Evaluation of effect of these particular Ig on the phosphorylation of HER expressing cells is currently underway.

Task 3: Months 1-48 To determine whether responses to HER2 are beneficial or detrimental, stratifying for function.

These studies require sera from large cohorts of breast cancer patients. The HER2 status needs to be known for all and all need to be treated with the same regimen. A major problem in breast cancer research in general and our work specifically, is a lack of sera from breast cancer patients linked to evaluable clinical databases. Large volume, well-defined, breast cancer clinical databases with long term follow up and corresponding serum samples are essential for answering many important questions about breast cancer etiology, prevention, diagnosis, and treatment and are sorely lacking at this time. We explored all of the major oncology cooperative groups as well as several cancer centers well known for large volume breast cancer therapy trials and were surprised at the lack of availability of banked sera on defined patients. We were not able to adequately determine the role of serum Ab to HER2 in the evolution of malignancy and the correlation between existent immunity and outcome, because of a lack of available banked sera on well defined patients.

Therefore, to answer the questions in Aim #1 Task #3 we set up a serum bank for the National Surgical Adjuvant Breast and Bowel Project (NSABP). We began collecting sera as of the date of 3/1/97. The NSABP maintains a comprehensive clinical database on study participants, but has not previously collected sera, with the exception of their cancer prevention trial called P-01. Funds from the current grant are not going to fund the serum bank, but will be used to ask specific questions posed on the grant as to the role of HER2 Ab on outcome. In addition, with the NSABP, we have initiated a study specifically designed to determine whether responses to HER2 are beneficial or detrimental. A study of antibody responses to HER2 will be performed in the context of NSABP B-27. Briefly, protocol B-27 is designed to determine whether 4 cycles of pre-operative or post-operative Taxotere given after 4 cycles of pre-operative Adriamycin (A) and cyclophosphamide (C) will more effectively prolong disease free survival and survival than 4 cycles of pre-operative AC alone. Protocol B-27.1 has been designed to obtain and analyze serum from B-27 patients for the presence of HER2 circulating ECD and antibody and to correlate these factors with tumor response to pre-operative chemotherapy and survival.

Protocol B-27.1 requires patient blood to be drawn by the NSABP pre-therapy, post neo-adjuvant chemotherapy, post surgery, at 12 month follow up and at the first relapse. The sera is being processed and stored for evaluation of HER2 antibody. Both fresh frozen and paraffin-embedded tissue specimens are being collected and stored at the NSABP headquarters. The NSABP database includes information on demographics, risk factors, family history, clinical and pathologic factors, characteristics of tumor, treatment and outcome. Sera collection continues at the time of this

progress report. Serial serum is beginning to be collected on the same individuals. All patients are receiving the same adjuvant chemotherapy regimen. Thus, the variable of HER2 reactivity can be analyzed. Stored sera will be examined in batches for Ab to whole HER2 protein, ICD and ECD. The function of ECD reactive Ab will be determined. Circulating serum ECD levels will be measured. The level of HER2 overexpression on primary tumor will be determined by immunocytochemistry on tissue blocks by NSABP reference pathologists. The NSABP statistical group will provide correlations.

Task 4: Months 1-48 To determine whether Ab varies predictably with therapy and recurrence.

Levels of antibody might correlate with important clinical parameters. Thus, following levels of Ab might provide information useful for decision making. When tumor progresses or relapses, the increase in HER2 antigen load might be expected to stimulate rising titers. A rise in Ab titer might serve as a harbinger of relapse. While the study described in Task 3, NSABP 27.1, will give prognostic information concerning the correlation of HER2 specific antibody response and survival, sufficient time points of sera will be collected that we may be able to discern whether HER2 antibody levels vary predictably with recurrence. The patients included in the study above will have HER2 protein overexpression assessed on their primary tumor as well as circulating levels of ECD, shed HER2 protein, measured. Therefore, antibody data collected on these patients will allow the comparison of rise in antibody titer to the measurement of a more "classic" serum tumor marker; shed ECD protein.

In addition, we had begun to follow newly diagnosed HER2 positive breast cancer patients here at the University of Washington (UW). Too few patients of different stages were available on long term follow-up to continue those studies. The patient base at the UW often consists of patients who are undergoing specialized treatment and eventually return to their private oncologist in the community. We have initiated collaboration with Dr. Saul Rivkin at the Tumor Institute at Swedish Hospital here in Seattle. Dr. Rivkin's group is responsible for the primary treatment and management of over 50 newly diagnosed breast cancer patients/year. In this study, we propose to measure the antibody levels against HER2 in patients with HER2-positive breast cancer, stages I-IV, at different times during their disease course. All new patients with breast cancer, seen at the Tumor Institute over the next 12 months, will be approached to participate. Antibody levels would be drawn at diagnosis and followed at 3 month intervals throughout the patient's treatment and follow-up. Correlation between HER2-specific antibody levels, treatment responses, disease progression, and prognosis will be assessed. The estimate time of follow-up is 3 years.

Task 5: Months 13-48 To determine whether immunity to HER2: (a) is present in patients with DCIS; (b) correlates with progression of HER2+ DCIS to HER2 negative invasive cancer; and (c) represents a possible marker for early cancer.

Studies of breast cancer biopsies show that HER2 levels are increased in the majority of DCIS specimens, but are not seen in atypia or dysplasia. Thus, overexpression of HER2 appears to be associated with malignant transformation and early neoplasia, but not benign proliferative diseases of the breast. This observation raises the question whether HER2 negative invasive ductal breast cancer arises from HER2 positive DCIS and whether HER2 immunity plays any role in immunoselection of progressive HER2 negative invasive ductal cancer from HER2 positive DCIS. Evaluating the immune response of newly diagnosed patients with DCIS and comparing that response to newly diagnosed patients with invasive breast cancer would lead to a better understanding of the interaction between the immune system and HER2 positive cancer.

To date, two collaborations have been formed to collect material specifically on patients with DCIS. Unfortunately, the finding of isolated DCIS without a focus of invasive breast cancer on re-excision has been rare. We have collected only 6 samples in the last 12 months from patient who have pure comedo type DCIS at these two centers; Dr. Ken Bertram, Madigan Army

Hospital, Tacoma, WA and Drs. Robin Ward and Nick Hawkins at St. Vincent's Hospital and the University of New South Wales, Sydney, Australia. We recently started a collaboration with Dr. Carol J. Fabian at the University of Kansas Medical Center. Dr. Fabian is conducting a Phase I study of a new selective estrogen receptor modulator (SERM) through the Chemoprevention Branch of the NCI. Patients with DCIS, T1, and T2 breast cancer will receive the agent for the interval between initial diagnostic biopsy and subsequent definitive surgical resection. HER2 antibody levels will be assessed on these patients as well as HER2 overexpression in DCIS or invasive breast cancer. In addition, the group will collect sera on all patients with DCIS evaluated, even those who do not enter the study. It is hoped collecting samples within the context of a Phase I treatment study will increase the population available for sera collection.

Finally, sera from NSABP P-01 protocol provides a unique resource to address these issues. Protocol P-01 is designed to test the hypothesis that long-term treatment with tamoxifen is effective in preventing invasive breast cancer. Serum is being collected on 16,000 individuals. It is projected that 325 of the subjects will develop breast cancer over the next 8 years. By examining serial sera for Ab at the time of diagnosis of malignancy, at fixed future time points and at the time of entry onto the protocol it should be possible to determine whether immunity predates diagnosis. The prevalence of Ab to HER2 will be too low to provide a general screening assay for breast cancer.

Task 6: Months 1-48 To determine whether immunity to HER2 correlates with outcome of 2B1 bispecific Ab therapy.

We have been evaluating the development of HER2 specific antibodies in patients who have been treated with the bispecific antibody MDX-210. MDX-210 binds simultaneously to type I Fc receptors for immunoglobulin G (IgG) and to the HER2 protein. In over 35 evaluations performed in patients who received either the murine or humanized version of the antibody we have detected no increase in HER2 antibody in patients who had low level pre-existent antibody nor generation of new HER2 antibody responses. It is our conclusion that, at least with this construct, endogenous immunity to HER2 is not generated. Thus, endogenous immunity to HER2 can not be evaluated as a prognostic factor in these studies.

Specific Aim # 2: To examine CD4+ T cell immunity to HER-2/neu.

Task 7: Months 1-36 To develop in vitro priming with dendritic APC to generate HER2-specific CD4+ T cells and to identify the epitopes recognized.

Studies on methods to prime in vitro are detailed in Specific Aim 3. Work described in the previous progress report described methods of in vitro priming using dendritic cells. We have begun to establish bulk culture techniques using PBMC from patients with HER2 positive breast cancer stimulating in vitro with peptide which we have defined as potentially subdominant epitopes and assessing whether these lines respond to corresponding HER2 proteins. Figure 2 shows proliferative activity of a line generated using purified CD4+ T cells and two 15 mer peptides derived from HER2. Peptide 776.15 and 971.15 had been shown in screening assays to elicit responses in patients who had HER2 positive tumors. After 2 IVS both peptide and protein specific T cells are measurable for 776.15, but not for 971.15. Several putative class II epitopes are currently being evaluated in this fashion.

In addition to bulk culture techniques, cloning the cells directly from the patients have resulted in the generation of HER2 specific T cells. CD4+ T cells were purified from a patient with a HER2 overexpressing tumor and cloned by limiting dilution with recombinant ECD protein. 12 ECD protein specific clones have been established from this patient. Evaluation of TCR shows the

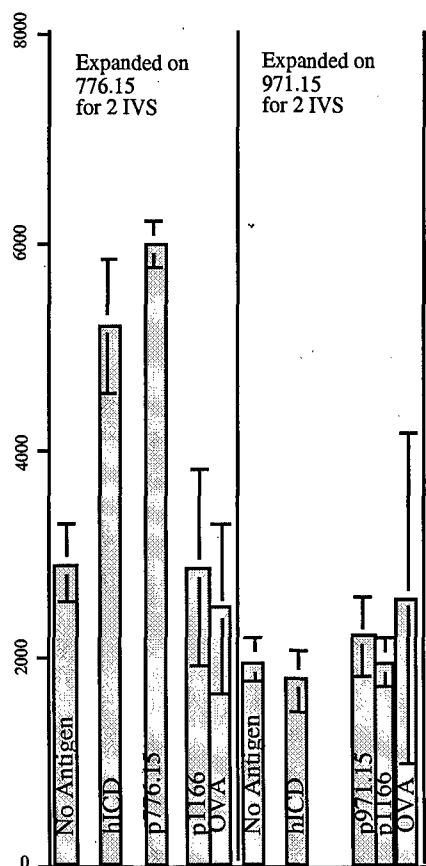


Figure 2. HER2 peptide specific and protein specific T cell lines can be established in HER2 positive patients. 3×10^7 PBMC derived from a patient with a HER2+ tumor were incubated with 776.15 or 971.15 peptide and IL-2 for 10 days. The lines were restimulated with peptide and autologous PBMC and analyzed 10 days later. T cell lines were analyzed for response to recombinant ICD protein, OVA as an irrelevant protein, p1166.15 as an irrelevant peptide and included their stimulating peptide. Results are reported as mean and standard deviation of 6 replicate wells.

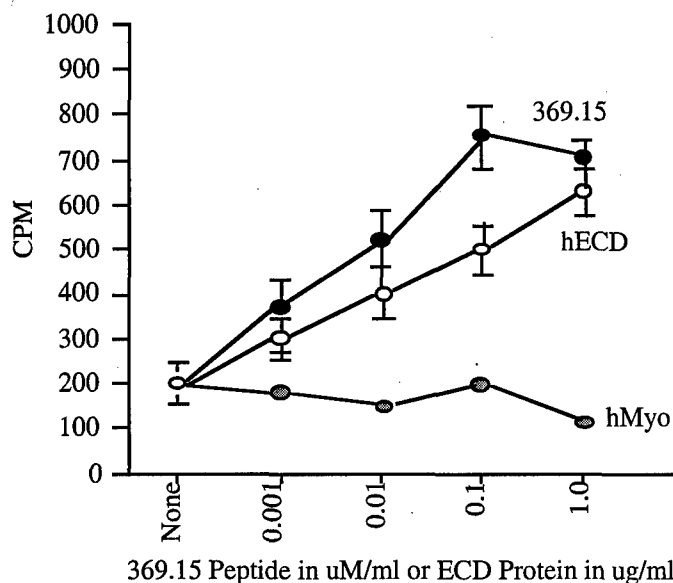


Figure 3. HER2 specific CD4+ T cell clones can be established on HER2 positive patients. Data is shown from a CD3+CD4+ T cell clone. The T cell responds specifically to recombinant human ECD protein, but not to human myoglobin used as an irrelevant control. The epitope recognized by the T cell is p369.15, a peptide in the ECD.

clones are all $\alpha\beta$. These clones are being analyzed for their epitope specificity using the 15 mer peptides predicted by in vitro testing to be potential class II epitopes. Data from one clone analyzed in such a fashion is shown in Figure 3. The clone responds specifically to hECD and not to human myoglobin used as an irrelevant protein and also responds to 369.15 a 15 mer derived from the HER2 protein.

These techniques will allow validation of peptides as naturally processed CD4+ T cell epitopes derived from the HER2 protein sequence as well as lay the foundation for the initiation of the treatment of HER2 overexpressing tumors with the transfer of competent T cells which recognize the HER2 protein.

Task 8: Months 13-48 To determine whether Th immunity to HER2 can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2.

We are currently in the process of identifying patients with HER2 positive tumors and approaching them to undergo leukapheresis. These are patients who have no detectable baseline immunity to the HER2 protein. It is our hypothesis that HER2 immunity can not be detected in these patients due to lack of quantitative measure of T cells response. Therefore, we have spent time developing in vitro techniques that may be more sensitive in determining baseline HER2 precursor frequency for cytokine producing T cells via Elispot. Elispot assays rely on determination of cytokine

expression in individual cells and has been shown to be highly sensitive, quick, relatively easy and uses only small amounts of PBMC. In initial experiments we used Elispot to evaluate normal donors responding to Flu antigens (Figure 4). The data demonstrate that flu specific T cell responses can be readily and reliably detected using the Elispot method.

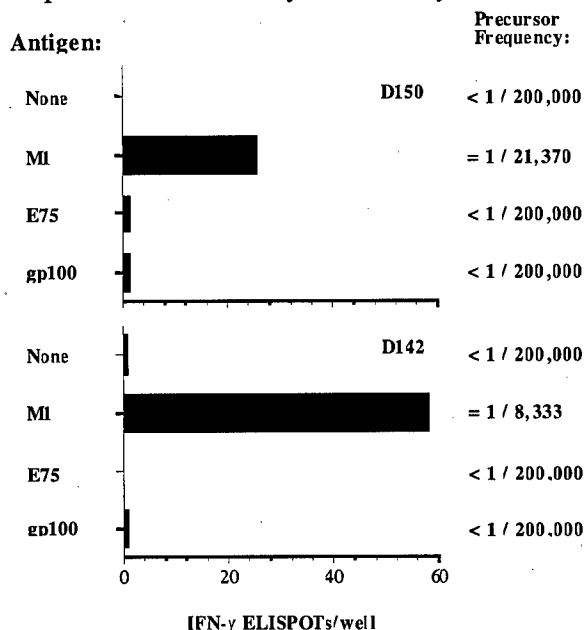


Figure 4: Elispot analysis of the flu M1 peptide specific precursor frequencies of 2 normal HLA-A2 donors. Precursor frequency was accessed against M1, a flu matrix A2 binding peptide, E75 α HER2 A2 peptide, and gp100, an A2 peptide found in melanoma.

In addition, a potentially more accurate measure of precursor frequency of CD4⁺ T cells may be in measuring the cells quantitatively using FACS analysis. Proliferative assays for responses in these patients revolve around proliferation and cytokine secretion. Very little is known of cytokine profiles of antigen specific human CD4⁺ T cells, and the existence of polarized Th1 and Th2 responses has been documented in very few human situations. To evaluate cytokine profiles of HER2 specific T cells, we have been evaluating cytokine secretion by both the Elispot method and the FACSImmune assay. The FACSImmune assay detects antigen induced synthesis of cytokines by T cells using flow cytometry which allows the simultaneous evaluation of the cells for an additional cell surface marker, in this case, the expression of CD4.

As shown in Figure 5, p369 induced a weak but significant IFN response from patient T cells after expansion in vitro on antigen for 28 days (2 stimulation cycles). The same cell line generated strong IL-4 (Figure 6) and TNF responses but no IL-10 (Figure 7).

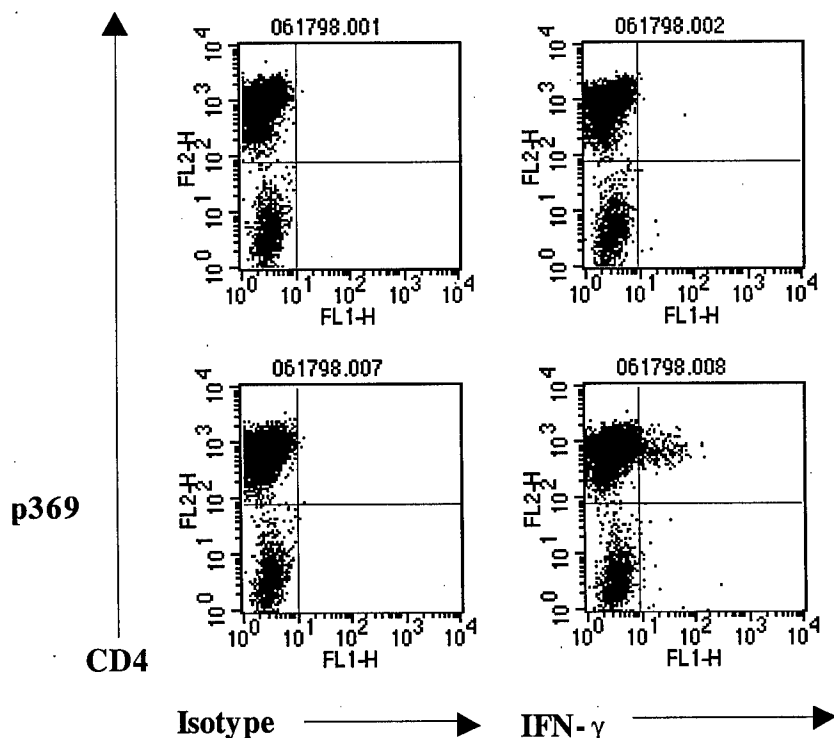


Figure 5: IFN FACSImmune assay of p369 specific T cells from a HER2 positive patient.

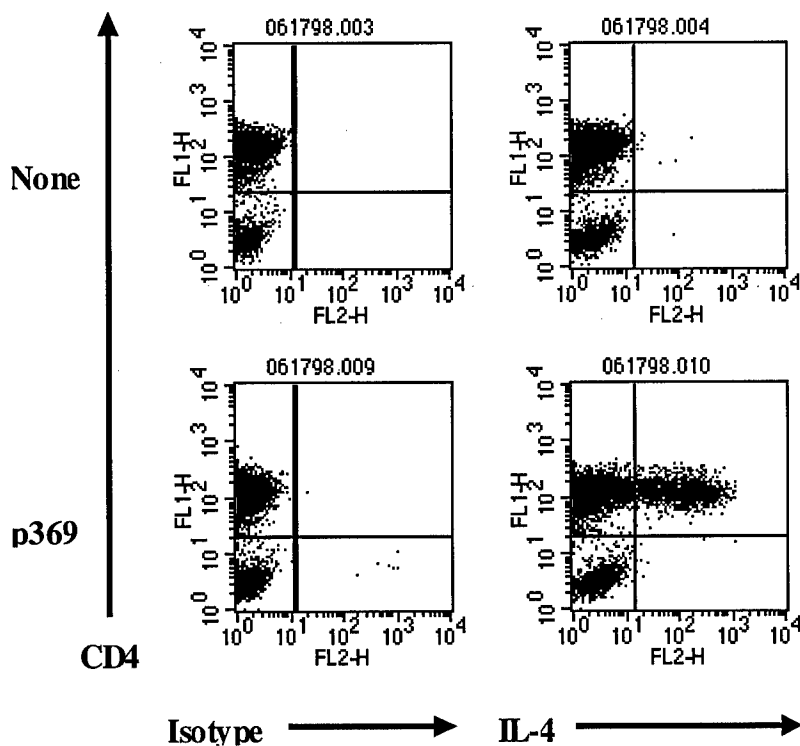


Figure 6: IL-4 FACSImmune assay of p369 specific T cells from a HER2 positive patient.

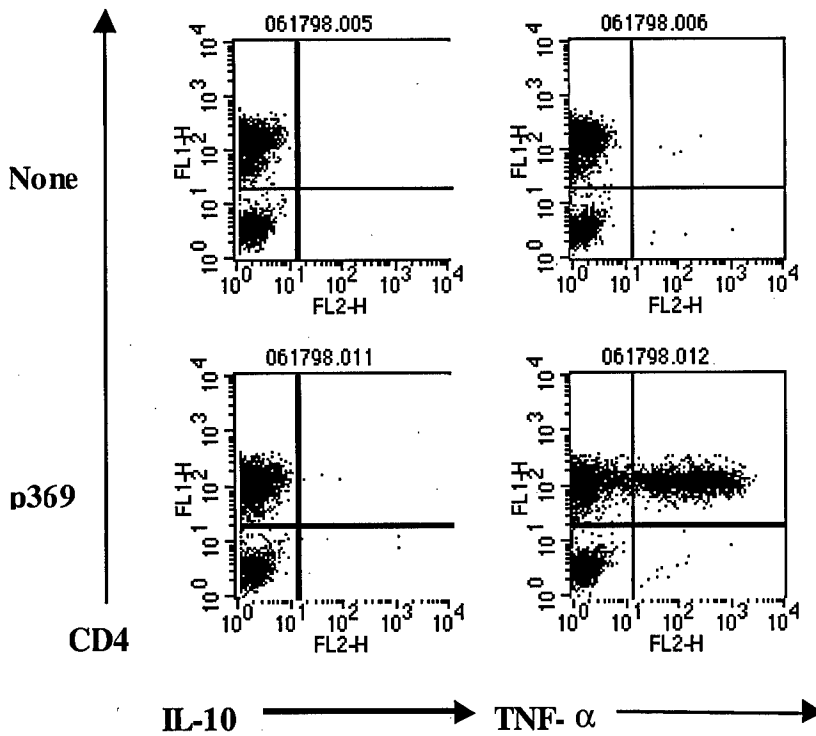


Figure 7: IL-10 and TNF FACSImmune assays of p369 specific T cells from a HER2 positive patient.

The development of more sensitive measures of the HER2 specific immune response will result in a more precise identification of patients who have little or no baseline immunity to HER2 as well as establish the clinical monitoring systems to assess the HER2 specific immune response after any antigen specific immunomodulation.

Task 9: Months 13-48 To determine the prevalence of CD4+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse.

This task requires serial assays. Assessment of serial assays has required the development of assays that are quantitative and statistically based. The challenge is designing quantitative assays while using only a limited amount of patient material. In work described in previous progress reports, HER2 specific T cell proliferation is assessed by using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors. This method is based on Poisson distribution, a more appropriate method for identifying randomly distributed responding cells of low number in replicate wells. A cutoff value of the mean and 3 standard deviations of the no antigen wells is used to score a well as responding or non-responding. All antigens are set up in 24 well replicates. Responses can be quantified as the number of responding wells of 24 well replicate. Using this cutoff value, one has a 95% confidence interval that a well is positive due to the specific antigen added. This assay system allows for quantification of HER2 specific T helper responses after each sequential immunization and uses a limited number of cells. Assays such as those described above require less PBMC and may be more sensitive. We will initiate long term studies evaluating T cell characterization once we have compared the quantitative assays described and defined the sensitivity and specificity of each in a limited number of patients.

Task 10: Months 13-48 To determine whether CD4+ responses modulate the biology of autologous tumors in vivo.

Studies will be designed based on the data obtained in Task 8 and 9 above.

Specific Aim # 3: To examine CD8+ CTL immunity to HER-2/neu.

Task 11: Months 1-48 To develop in vitro priming with dendritic APC to generate HER2 specific CD8+ T cells and to identify the epitopes recognized.

We have embarked on studies to determine the most efficient, physiologically relevant, means of generating HER2-specific CTL by priming in vitro. Several approaches are being studied including experiments 1) to elicit CTL by in vitro priming with peptides and dendritic cells (DC), 2) to elicit CTL by in vitro priming with DC transfected with either DNA or RNA encoding HER2, and 3) to elicit CTL by in vitro priming with DC infected with recombinant virus encoding HER2. Each method has distinct advantages and disadvantages. Each has yielded encouraging results. The previous progress report outlined our work on using DC for the generation of HER2 specific lines. Over the last year we have focused our efforts on generating autologous targets using "immortalized" APC. While the work on DC generation continues, the strategies outlined below will offer long term APC lines for individual patients and the technology may be more conducive to transfer to a clinical trial format for the analysis of a vaccine or adoptive immunotherapy trial.

We have explored the use of generating autologous targets from individual patients using various viral constructs. Validation of the ability to lyse HER2 expressing cells is best when proven in an autologous system. We have constructed vaccinia virus and tested ability of HER2 specific CTL above to lyse EBV transformed B cells (BLCL). As shown in Figure 8, CTL that are primed in vitro to H7, a peptide that has been shown to be a natural HLA-A2 epitope of the HER2 protein, can specifically lyse targets cells infected with HER2 vaccinia, but not vaccinia expressing an irrelevant antigen (P502S). The potential ability to use patients' cells expressing HER2 as well as knowledge of additional epitopes will be important in the eventual analysis of the immune responses in patients immunized with HER2 vaccines.

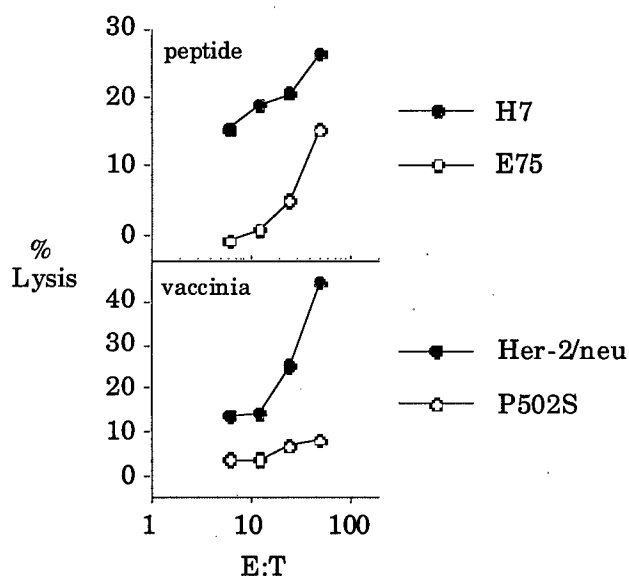
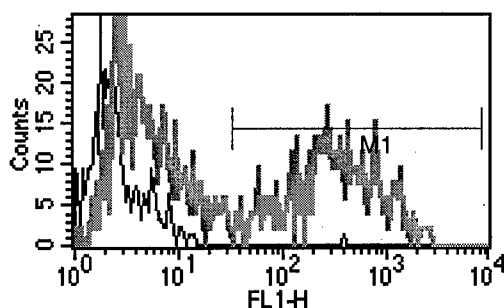


Figure 8: CTL primed in vitro to HER2 H7 peptide lyse peptide loaded (top) and HER2 vaccinia infected (bottom) target cells.

The use of in vitro priming of human CTL with peptides has proven very useful to demonstrate that HER2 is immunogenic, processed and presented by breast cancer cells and processed and presented by class I MHC molecules. However, immunization to cells constructed to express HER2 might be more efficient and lead to better vaccine strategies. Rather than using priming in vitro with synthetic peptides, the alternative strategy would use antigen-presenting cells (APC) that have been transfected with the cDNA encoding for HER2. Several approaches are under study, including the use of retroviral vectors to transfer HER2 cDNA into fibroblast cell lines, the gene gun to transfect dendritic cells, and finally, the use of vaccinia and adenovirus to get high level expression in dendritic cells and monocytes.

Fibroblasts were transduced with a HER2 retroviral vector and drug selected. This process rapidly leads to the generation of a stable cell line with high level expression of the transferred gene. Furthermore, these cells can be retransduced with another retroviral vector. We have generated HER2 transduced fibroblasts which were retransduced with a B7.1 retrovirus. Approximately 30% of the fibroblasts expressed B7.1 as a result of this procedure, in the absence of any drug selection. This level can be readily increased to 100% with drug selection. These fibroblasts will allow comparisons to peptide loaded dendritic cells for in vitro priming of HER2 specific CTL. In addition to testing retroviral transduced fibroblasts, experiments are under way to introduce candidate cDNAs into normal human B cells and dendritic cells. Introduction into fibroblast is easiest. Thus, we started there. However, both B cell and dendritic cells are better APC.

Fibroblast cell lines have been generated that express antigens and costimulatory molecules such as HER2 and CD80 (B-7.1). This was accomplished readily using retroviral vectors. The same was also accomplished using normal human B cells that were activated with CD40L. Such B cells are actively dividing and as can be seen in Figure 9, they are highly transducible with retroviral vectors. Without any drug selection, 43% of the B cells were effectively transduced with the EGFP retrovirus. To determine the optimal method for introducing HER2 into APC, vaccinia and adenoviral vectors are being constructed and will be tested.



CD40L B cells

Marker	% Gated
All	100.00
M1	43.21

Figure 9: Transduction of CD40L activated human B cells with EGFP retroviral vector

The advantage of cDNA, RNA and retroviral transduction of APC is that there will not be concurrent expression of weak tumor antigen with strong, immunodominant, foreign antigen. However, the best way to get optimal expression of tumor antigen is still with viral vectors. To test the use of viral vectors in the HER2, we are constructing vaccinia virus and adenovirus. Two vectors are needed with non-cross reactive foreign antigens, so that one can be used for priming and the other for re-stimulation and/or target. A number of vaccinia (Table 1) and adenoviral (Table 2) vectors have been constructed. Figure 10 shows HER2 expression by DC infected with recombinant HER2 vaccinia virus. Priming experiments will be performed with the non-cross reactive expression vectors.

Table 1: Vaccinia Virus Constructs

Vaccinia Virus Constructs	Protein Expressed		Protein Expression Verification
VV30	human full length Her-2/neu		Completed FACS
VV21	H2N ECD plus signal peptide		Completed Western
VV22	H2N ECD no signal peptide		Completed Western
VV23	H2N ICD		Completed Western
VV49	EGFP		Completed Fluorescence microscopy

Table 2: Adenovirus Constructs

Adenovirus Constructs	Protein Expressed	Protein Expression Verification
Ad/ICD	Her-2/neu Intracellular domain	Completed Western
Ad/ECD	Her-2/neu extracellular domain	Completed To be tested
Ad/EGFP	enhanced green fluorescent protein	Completed To be tested

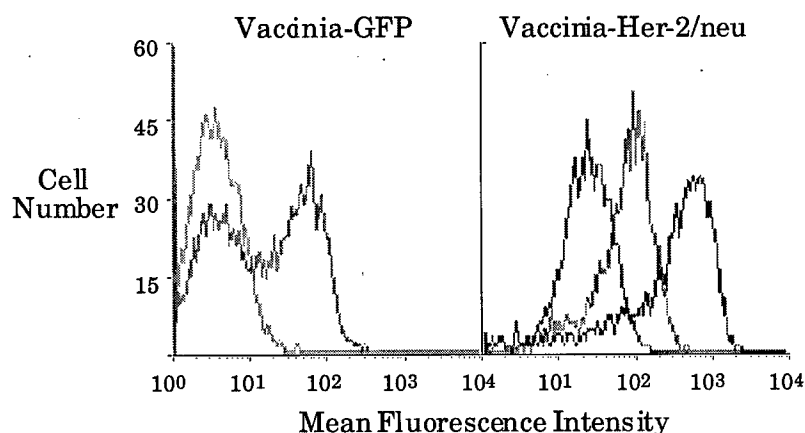


Figure 10: Dendritic cells were generated from human PBMC adherent cells by culture in GM-CSF+IL-4 for 7 days, followed by infection with the indicated virus for 1 day (GFP) or for 5h (HER2 green) or 16h (HER2 pink).

Task 12: Months 1-36 To determine whether CD8+ CTL immunity can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2.

The same techniques described in Specific Aim 2 are also being evaluated for this Aim in terms of CD8+ T cells. Previously, our ability to detect HER2 specific CTL was limited to HLA-A2 as the peptide epitopes for that HLA molecule were the only ones well characterized. We have also begun to evaluate CTL responses in other HLA types outside of HLA-A2. The majority of work searching for CTL epitopes has been performed for HLA-A2.1. The allele covers at most 40% of the population. We have begun a search for peptides restricted by other alleles. A set of rules for peptide binding to other HLA alleles are being established regarding peptide selection that are similar to those being used for HLA-A2.1. Doing so will provide greater flexibility for peptide priming CTL in vitro. HLA-B44 was selected for this purpose for the following reasons, (a) HLA-B44 is the most frequent HLA-B allele in the Caucasian population. According to a recent report on the HLA haplotype frequency in a 1.35 million person sample of the North American population, B44 was found in 13.5% of the Caucasians, comparing to 28.7% for HLA-A2. In the more homogeneous European population, that frequency is even higher. The Spanish, for instance, have a frequency of 28% for HLA-B44, (b) B44 is dominated by only 2 highly similar subtypes, B*4402 (33%) and B*4403 (66%), which differ from each other by only one residue, (c) The motif for the two B44 suballeles is almost identical and it is much less ambiguous than the HLA-A2. It requires Glu at P2 and an aromatic residue (Tyr, Phe or Trp) at P9 or 10 as anchor residues. Although synthetic peptides with Asp at P2 or other hydrophobic residues at P9 can bind B44, Glu and Tyr/Phe dominate the naturally eluted peptides, (d) The fact that Glu is required for the P2 and Arg and Lys are preferred for secondary residues makes it technically simple to synthesize and purify B44 restricted peptides. Indeed, most of them are soluble in PBS, whereas many of the A2 peptides are quite hydrophobic and difficult to solubilize.

The B44 restricted peptides in Table 3 below have been constructed. Priming experiments will determine which, if any are immunogenic and naturally processed epitopes. Vaccinia infected HLA-B44+ BLCL will be used to validate that the epitopes are naturally processed.

Table 3: HER2 derived peptides predicted to bind to HLA-B44.

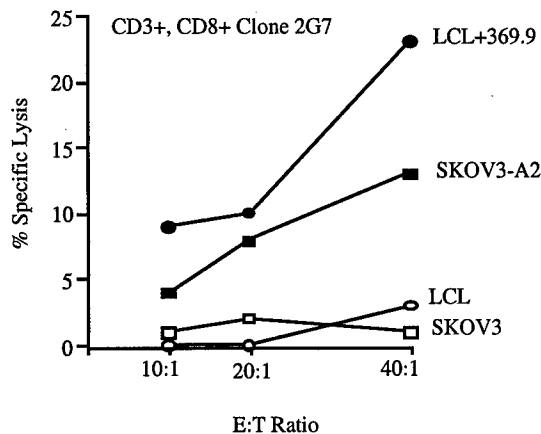
HER2 B44-1	QDIQEVQGY
HER2 B44-2	LEEITGYLY
HER2 B44-3	SEGAGSDVF
HER2 B44-4	LESILRRRF
HER2 B44-5	FETLEEITGY
HER2 B44-6	QEFAGCKKIF
HER2 B44-7	EEYLVPQQGF

Task 13: Months 37-48 To determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse.

We have yet to initiate long term studies on quantitation of HER2 specific CTL responses in patients with breast cancer. Once the studies described above reveal the most robust method of analysis, we will institute these investigations.

Task 14: Months 13-48 To determine whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor and can be expanded in vitro to the extent needed for adoptive therapy.

We have begun to clone CTL specific for HER2 from the PBL of patients with HER2 positive tumors. We have begun to study 3 patients with HER2 positive tumors and HLA-A2 background in detail. As an example, data is shown on one patient. CD8+ T cells were isolated and cloned in a limiting dilution fashion in the presence of HLA-A2 binding peptide 369.9 a natural epitope of HER2 and IL-2. 21 p369.9 peptide specific CTL were generated, all are CD3 and CD8+. Data is shown in Figure 11 for one clone, 2G7. The T cell specifically lyses autologous BLCL loaded with the HER2 peptide as compared to BLCL alone, but also shows some activity against an A2 transfected HER2 overexpressing cell line (SKOV3-A2). Interestingly, TCR analysis of the 21 clones generated revealed 19 were $\alpha\beta$ and 2 were $\gamma\delta$. 6 of the clones have been evaluated for cytokine secretion (IFN γ) and 4 secreted IFN while 2 did not. Other cytokines being tested are TNF, IL-4 and IL-10.



The generation of CD8+ HER2 specific lines and clones will allow the assessment and characterization of in vitro activity for potential in vitro correlations as well as develop the in vitro expansion techniques needed for expansion of HER2 specific T cells for potential use in treatment.

Figure 11. CD8 CTL clones specific for HER2 can be isolated from patients with HER2⁺ tumors

CONCLUSIONS:

The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. The studies thus far have validated that immunity to HER2 exists and can be augmented by manipulation in vitro. The studies have thus far provided great assistance in developing HER2 vaccines as well as lay the foundation for the development of HER2 specific adoptive immunotherapy.

Studies of antibody responses confirmed that Ab immunity to HER2 can be detected in the sera of some patients with breast cancer and is correlated with antigen expression. The increased frequency of Ab in patients with HER2+ cancer strongly implies that immunity develops as the result of the overexpression of HER2 on breast cancer, i.e., some patients become immune to their own cancers. The confirmation that some patients become immune to their own cancers gives great credence to the concept that vaccines will be able to elicit substantial immunity to HER2.

HER2 is a functioning growth factor receptor. Ab to HER2 in some patients was directed against the ECD and was able to perturbate function. This remarkable finding strongly implies that the immune response to HER2 might directly alter growth characteristics and outcome in patients with breast cancer. Whether patients with HER2+ cancers and functional Ab to HER2 survive longer as a result of Ab is being pursued. These studies may eventually provide evidence of substantial and important host tumor interactions.

The correlation between antibody and breast cancer shows that antibody responses have the potential to serve as tumor markers for detecting breast cancer. That hypothesis is being pursued. Also being pursued is the hypothesis that changes in level of Ab can detect early relapse.

The major question of whether existent immunity to HER2 relates to improved survival is being addressed using sera collected from the NSABP adjuvant breast cancer trials. Sera is drawn at the time of diagnosis and all patients are receiving the same adjuvant chemotherapy regimen. Thus, HER2 reactivity can be analyzed as an independent variable.

Finally, HER2 immune sera provides an excellent reagent to screen for other novel breast related immunogen proteins.

Studies of CD4+ T cell immunity to HER2 confirmed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy given that HER2 is an abundant soluble protein, i.e., the extracellular domain (ECD) is shed. In animal models CD4+ T cells can be effective against abundant soluble proteins.

For vaccine development identification of peptide epitopes is important. Extensive empirical testing of putative helper T cell epitopes in vitro resulted in the identification of potential vaccine candidate epitopes. We have developed the culture system for the expansion of peptide specific T cell lines and have shown that some putative epitopes can respond to protein and other don't.

We are testing highly sensitive and reproducible assays for evaluating CD4+ and CD8+ T cell responses to HER2. These assays are being used in the current studies to identify, quantify and follow existent immune responses to HER2.

Studies of CD8+ T cell immunity to HER2 confirmed that CD8+ CTL can be primed to HER2 peptides in vitro and that primed peptide specific CTL can lyse HER2 positive cancer cells. We have embarked on studies to determine the most efficient, physiologically relevant, means of generating HER2-specific CTL by priming in vitro including in vitro priming with peptides, dendritic cells transfected with either DNA or RNA and dendritic cells infected with recombinant

virus encoding HER2. Each method has yielded encouraging results, but with different advantages and disadvantages. No method has solved the essential question of how best to prime and how to prime reproducibly.

A major issue is whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor. We have been able to generate HER2 specific lines and clones and characterize them as to their potential function. Finally, we have begun the experiments necessary to determine whether HER2 specific T cells can be expanded and whether such cells can be expanded with maintenance of function to the extent presumed necessary for therapy.

CURRICULUM VITAE

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Professional Experience:

1986 -1989 Residency in Internal Medicine, University of Illinois, Chicago, Illinois

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1993 to 9/1994 Acting Instructor, Division of Oncology, University of Washington, Seattle, Washington

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Honors and Responsibilities:

Spirit of Creighton Award, 1981

Michaelson Scholarship for Research in Cystic Fibrosis, 1984, 1985

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Intern of the Year, University of Illinois, 1987

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Recipient, Clinical Oncology Fellowship, American Cancer Society, 1990-1991

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Clinical Investigator Award, National Cancer Institute, 1995-2000

Recipient, FIRST Award, National Cancer Institute, 1995-2000

Science in Medicine, New Investigator Lectureship, University of Washington, 1995

Chairman, Molecular Therapeutics Committee, Southwest Oncology Group, 1998-

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Physician Data Query (PDQ) Protocol Review Board, 1998-2001

Curriculum Vitae, Page 2

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U.S. Patents:

1. U.S. Patent# 5,801,005. Immune reactivity to HER-2/neu protein for diagnosis and treatment of malignancies in which the HER-2/neu oncogene is associated.
2. 08/625,101 (920010.448C7) Compounds for eliciting or enhancing immune reactivity to the HER-2/neu protein for prevention or treatment of malignancies in which the HER-2/neu oncogene is associated.

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Member, American Association of Immunologists (AAI)
Member, Women in Cancer Research (WICR)
Member, American Society for Clinical Oncology (ASCO)
Member, American Society of Gene Therapy
Member, Puget Sound Oncology Consortium (PSOC)
Member, Seattle Breast Cancer Program
Member, Southwest Oncology Group (SWOG)

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
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